Distribution of viral antigen and tissue lesions in persistent and acute infection with the homologous strain of noncytopathic bovine viral diarrhea virus

Elisabeth M. Liebler-Tenorio,[2] Julia F. Ridpath,[1,2] John D. Neill[2]

Abstract. Viral distribution and lesions were compared between calves born with persistent infection (PI) and calves acutely infected with the same bovine viral diarrhea virus (BVDV) isolate. Two PI calves from 1 dairy herd were necropsied. The PI viruses from these calves were isolated, characterized by sequencing, and found to be identical. This virus strain, designated BVDV2-RS886, was characterized as a noncytopathic (ncp) type 2 BVDV. To establish acute infections, BVDV2-RS886 was used to inoculate clinically healthy, seronegative calves which were 3 weeks to 3 months old. Nine calves received 10^6–10^7 tissue culture infective dose of BVDV2-RS886 intranasally. Four additional age-matched animals served as noninfected controls. Infected calves were necropsied at 3, 6, 9, or 13 days postinoculation (dpi). Viral antigen was detected by immunohistochemistry in frozen sections, and lesions were evaluated in hematoxylin eosin–stained paraplast sections. In the PI calves, a wide distribution of viral antigen was found in all tissues and was not associated with lesions. In the acutely infected calves, viral antigen was widespread in lymphoid tissues at 6 dpi but had been mostly eliminated at 9 and 13 dpi. Depletion of lymphoid tissues was seen at 6, 9, and 13 dpi and repopulation at 9 and 13 dpi. In 1 of the calves at 13 dpi, severe arteritis was present in lymph nodes and myocardium. This comparison shows that an ncp BVDV strain that causes no lesions in PI animals is able to induce marked depletion of lymphoid tissues in calves with acute infection. Therefore, the failure to eliminate PI cattle from a herd causes problems not only in pregnant cattle but may also affect other age groups.

Introduction

Infection of immunocompetent cattle with bovine viral diarrhea virus (BVDV) can cause acute bovine viral diarrhoea.[1] Most acute infections either with noncytopathogenic (ncp) or cytopathogenic (cp) BVDV are subclinical.[13,19] Severe disease with high mortality and hemorrhage may occur, especially with BVDV2.[6,9,24–26] Acute infection is followed by an immune response, which is reflected by the appearance of neutralizing antibodies. Acute infection of pregnant naive cows may result in the intrauterine infection of the fetus and the birth of calves with persistent infection (PI) with ncp BVDV.[10,18] These animals have a selective immunotolerance for the persisting ncp BVDV strain and do not mount an immune response against the persisting virus.[20] When PI calves are superinfected with a matching cp BVDV, they may develop fatal mucosal disease.[4,5]

Persistently infected cattle are very important for the transmission of BVDV because they shed continuously high amounts of ncp BVDV.[21] Experimentally, it was demonstrated that transmission from PI cattle was much more efficient than that from acutely infected cattle.[22] Therefore, most BVDV control programs include the elimination of PI animals. In this study, it is demonstrated that the same ncp BVDV strain that causes no lesions in PI calves will result in tissue lesions in acute infection. Thus, besides the potential transmission to pregnant cows and the consequences of fetal infections, it may also affect the health of other herd members.

Materials and methods

Animals. Two PI calves, acquired from the same dairy herd, were determined to be persistently infected on the basis of positive immunohistochemistry in skin biopsies and virus isolation from buffy coat samples on consecutive samples collected 3 weeks apart. They were clinically healthy and had no apparent developmental abnormalities. For the acute infection experiment, 13 clinically healthy, colostrum-deprived, 3-week to 3-month-old male calves of mixed breeds were used. The latter were negative for BVDV as determined by virus isolation from buffy coat followed by polymerase chain reaction (PCR)[27] and did not have antibodies to BVDV as determined by serum neuralization using BVDV 1 strain NY-1 and BVDV 2 strain 1373.

Virus. Bovine viral diarrhea virus was isolated from the buffy coat of the 2 PI calves (calves 14, 15).[27] Both viruses were ncp on the basis of propagation in cultured Madin Darby bovine kidney and bovine turbinate cells. A portion of the 5′-UTR region was amplified and sequenced as described previously.[26] The 5′-UTR sequences derived for the viruses

[2]From the Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA-ARS, PO Box 70, Ames, IA 50010 (Ridpath, Neill). Current address (Liebler-Tenorio): Federal Research Center of Virus Diseases in Animals, Naumburgerstr. 96a, 07743 Jena, Germany.

[1]Corresponding Author: Julia F. Ridpath, Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA-ARS, PO Box 70, Ames, IA 50010.
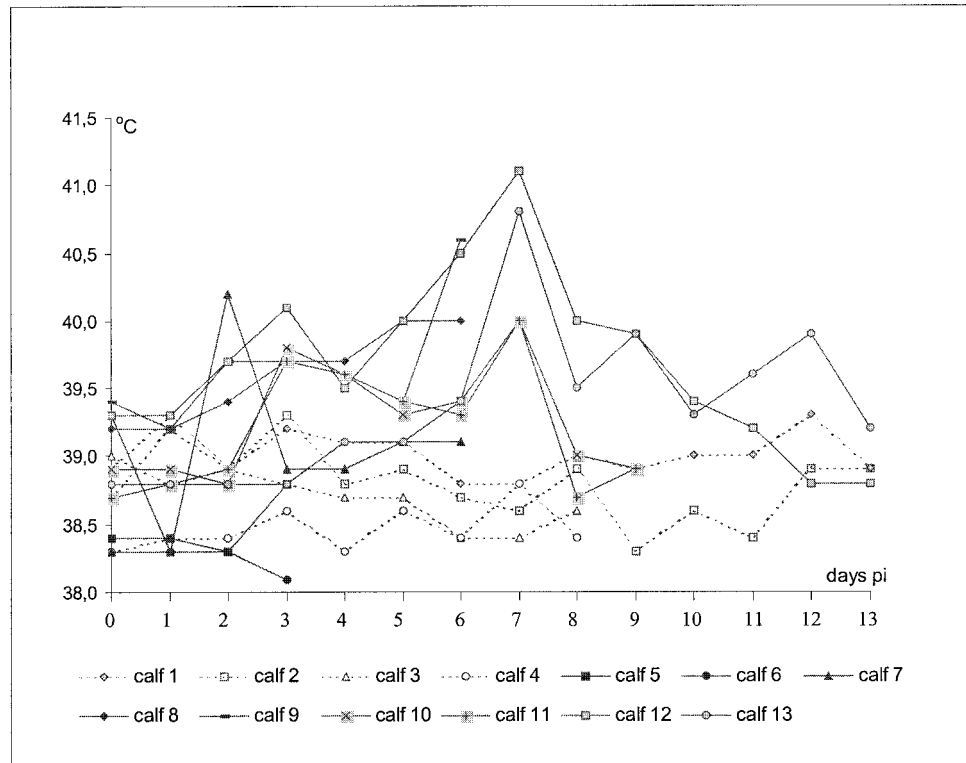
Figure 1. Body temperature after inoculation with BVDV2-RS886.

isolated from each calf were identical. The virus from calf 14 was designated BVDV2-RS886 and used as the inoculum for this experiment. The virus was used as inoculum after 4 passages in tissue culture (this includes the 2 blind passages used in the original isolation).

Experimental procedure. For the acute experimental infection, 9 calves (calves 5–13) received 5 ml containing 10^6–10^7 tissue culture infective dose (50%) of BVDV2-RS886 intranasally and 4 calves (calves 1–4) served as controls and were not inoculated. Body temperatures were monitored dai-
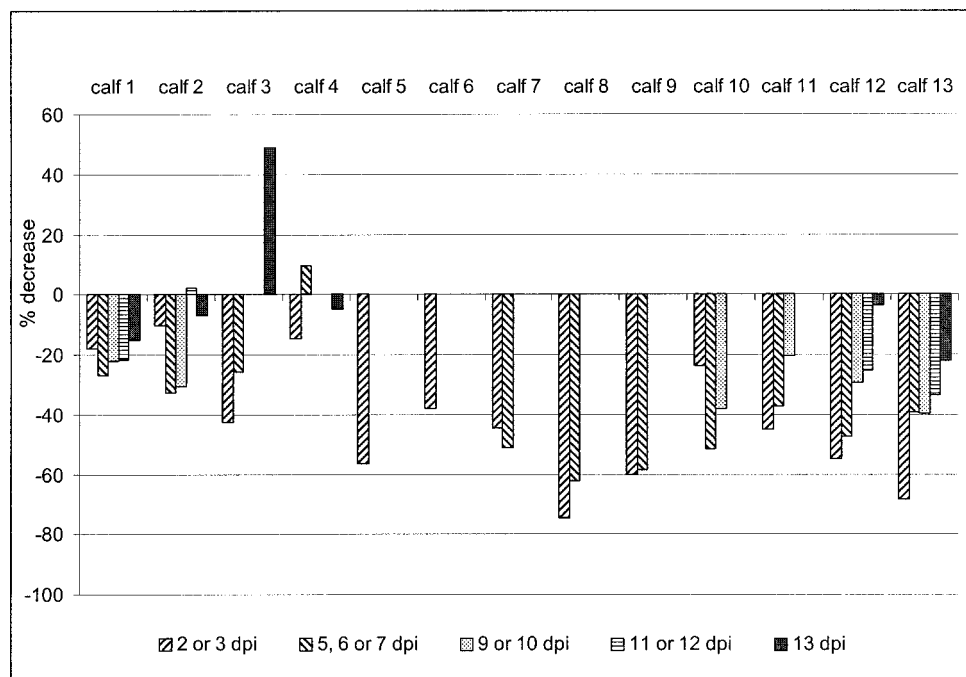

Figure 2. Decrease of lymphocyte numbers after inoculation with BVDV2-RS886.

Table 1. Organ and tissue distribution of antigen after inoculation with BVDV2-RS886.

Animal number	Control		3 dpi*		6 dpi			9 dpi		13 dpi		PI†	
	3	4	5	6	7	8	9	10	11	12	13	14	15
Lymphoid tissues													
Tonsil	−	−	+	+	+	ND‡	+	−	−	ND	−	+	+
Mandibular lymph node	−	−	+	+	+	+	−	−	−	−	−	+	+
Retropharyngeal lymph node	−	−	+	−	+	+	+	−	−	+§	−	+	+
Superficial cervical lymph node	−	−	−	−	+	+	+	−	−	−	−	+	+
Mesenteric lymph node	−	−	+	+	+	+	+	−	−	−	−	+	+
Spleen	−	−	+	+	+	−	+	+§	−	−	−	+	+
Thymus	−	−	−	−	+	+	+	ND	+	+	−	+	+
Bone marrow	−	−	−	−	−	−	−	−	−	−	−	+	+
Intestines													
Duodenum	−	−	−	−	+	−	−	−	−	−	−	+	+
Jejunum	−	−	−	−	+	−	−	−	−	−	−	+	+
Jejunum with Peyer's patch	−	−	+	−	+	+	+	−	−	−	−	+	+
Ileum with Peyer's patch	−	−	+	−	+	+	+	+	−	−	−	+	+
Ileocecal entrance	−	−	+	+	+	+	+	−	−	−	−	+	+
Colon with lymphoid nodule	−	−	−	+	+	ND	+	ND	−	−	−	+	+
Midcolon	−	−	−	−	+	−	+	−	−	−	−	+	+
Rectum with lymphoid nodule	−	−	+	+	+	+	+	ND	−	−	−	+	+
Upper digestive tract													
Oral mucosa	−	−	−	−	−	+	+	−	−	−	−	+	+
Parotis	−	−	−	−	−	ND	−	−	−	−	−	+	+
Esophagus	−	−	−	−	−	−	−	−	−	−	−	+	+
Rumen	−	−	−	−	−	−	−	−	−	−	−	+	+
Abomasum	−	−	−	−	−	+	−	−	−	−	−	+	+
Respiratory tract													
Nasal orifice	−	−	−	−	−	−	−	−	−	−	−	+	+
Turbinate	−	−	−	−	+	−	−	−	−	−	−	+	+
Trachea	−	−	−	−	+	−	−	−	−	−	−	+	+
Lung	−	−	−	−	+	−	−	−	−	−	−	+	+

* Days postinoculation.
† Persistently infected.
‡ Not done.
§ Only vascular walls.

ly. Blood samples were collected at the time of inoculation and then every other day. In these samples, the number of lymphocytes and thrombocytes was determined, and the buffy coat was examined for BVDV by virus isolation followed by PCR.[27] Inoculated calves were euthanized and necropsied at 3 (calves 5, 6), 6 (calves 7–9), 9 (calves 10, 11), and 13 (calves 12, 13) days postinoculation (dpi). Two of the control calves (calves 1, 2) were used for clinical parameters only and 2 (calves 3, 4) were necropsied at the end of the experiment. The PI calves (calves 14, 15) were also necropsied at the end of the experiment.

Histology and immunohistology. At necropsy, tissue samples were collected from 38 locations including lymphoid organs (tonsil, mandibular lymph node, retropharyngeal lymph node, subscapular lymph node, mesenteric lymph node, spleen, thymus), digestive tract (oral mucosa, esophagus, rumen, abomasum, duodenum, jejunum, colon, Peyer's patches in jejunum and ileum, lymphoglandular complexes in the colon and rectum, parotid gland, pancreas, liver), respiratory tract (nasal orifice, turbinates, trachea, lung), endocrine organs (thyroid, pituitary, pancreas, adrenal), uro-genital organs (kidney, testis), central nervous system (cerebrum, hippocampus, cerebellum), heart, skin, interdigital skin, and bone marrow. Two samples were collected from each site. One sample was fixed in 5% neutral-buffered formalin, embedded in paraplast, and sectioned; the other sample was snap-frozen in isopentane at −70 C for immunohistology. The paraplast sections were stained with hematoxylin and eosin (HE). Viral antigen was detected in cryostat sections by the indirect immunoperoxidase method, as described.[15] Monoclonal antibody (mab) BZ81, which recognizes an epitope on E2 and which had a high affinity for BVDV2-RS886, was used as the primary antibody.[a] Cell culture supernatant with mab BZ81 was diluted 1:2 in phosphate-buffered saline (pH 7.1) containing 1% Tween-20.[b]

Results

Clinical signs. The PI calves were clinically healthy and had normal body temperature. Lymphocyte and platelet numbers were in the normal range.
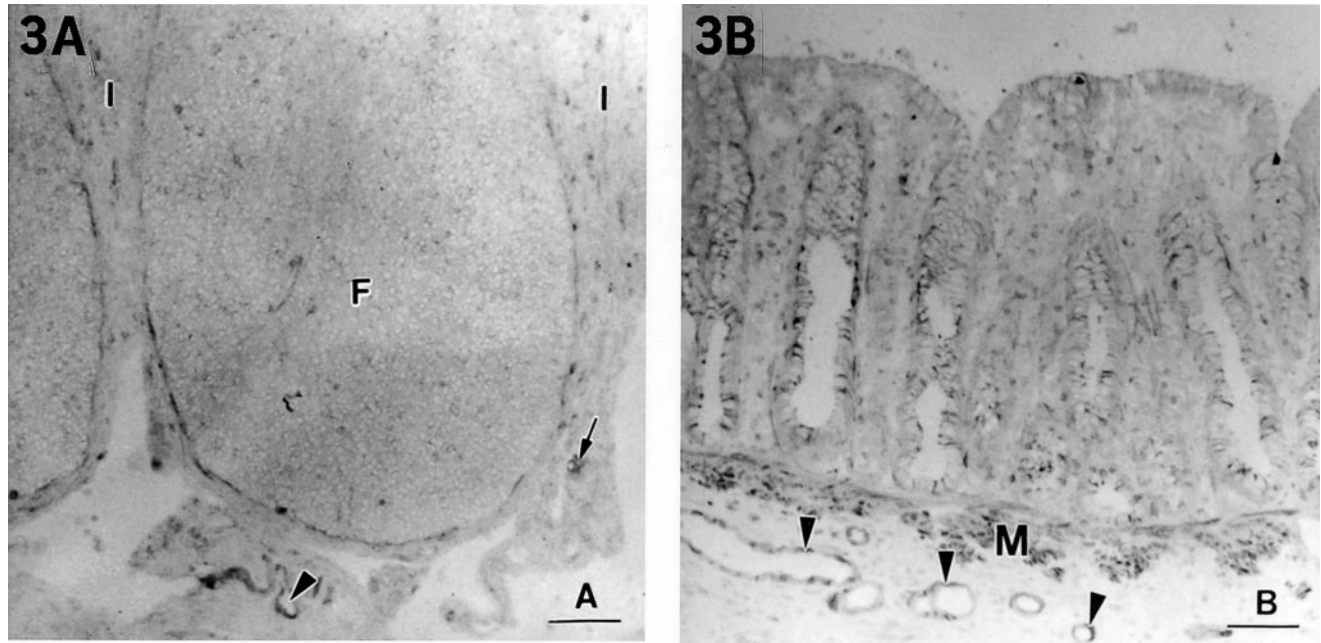
In acute infection, there was a mild temperature in-

Figure 3. Distribution of viral antigen in tissues of the PI calves. **A,** viral antigen is present in lymphoid follicles (F), interfollicular areas (I), and in vascular walls (arrowhead) and ganglia (arrow) of the submucosa. Ileal Peyer's patch. Calf 14, bar = 100 μm. **B,** in the intestinal mucosa, viral antigen is present in epithelial cells, cells in the lamina propria, myocytes of the muscularis mucosae (M), and vascular walls (arrowheads). Calf 14, bar = 100 μm.

crease at 2–3 dpi and a transient temperature peak of ≥40 C at 6 and 7 dpi (Fig. 1), but behavior and food intake were normal in all calves. A decrease in the number of circulating lymphocytes was seen initially at 2–3 dpi. The maximal decrease below baseline values ranged between 38% and 74% with recovery after 9–10 dpi (Fig. 2). There were no significant changes in platelet numbers.

Bovine viral diarrhea virus was isolated from the buffy coat of the PI calves. In acute infection, BVDV was initially detected by immunohistochemistry or virus isolation (or both) from buffy coat samples between 3 and 9 dpi.

Macroscopic findings. No lesions were seen at necropsy in the PI calves, in the calves from the acute infection, or in the control calves.

Distribution of viral antigen. In the PI calves, viral antigen was detected in all organs and tissues throughout the body (Table 1). To compare the findings in the PI calves with those in calves with acute infection, the distribution in lymphoid tissues and digestive tract is described in detail. In lymphoid tissues of the PI calves, viral antigen was found in all compartments (lymphoid follicles, interfollicular areas, paracortex, medulla, thymic cortex and medulla) in lymphocytes, cells with dendritic morphology, macrophages, sinus-lining cells, and vascular walls (Fig. 3A). In the digestive tract of these animals, viral antigen was detected in epithelial cells, mononuclear cells in the lam-

ina propria and submucosa, stromal cells, myocytes, and intramural ganglia (Fig. 3B).

In the acutely infected animals, viral antigen was initially seen at 3 dpi in lymphoid tissues, most consistently in the tonsil, retropharyngeal lymph node, mesenteric lymph node, spleen and mucosa-associated lymphoid tissue at the ileocecal entrance, and in the rectum (Table 1). The distribution of antigen-containing lymphocytes and cells with dendritic morphology was multifocal, predominantly in interfollicular areas and paracortex, in the periphery of lymphoid follicles, and less frequently within lymphoid follicles. In the tonsil, a few epithelial cells in the reticulate epithelium were antigen positive.

Among the acutely infected animals, the widest tissue distribution of viral antigen was seen at 6 dpi (Table 1). Viral antigen was present in lymphoid tissues and in the mucosa of the digestive and respiratory tract. In the lymphoid tissues, viral antigen was mostly limited to lymphocytes and follicular dendritic cells in lymphoid follicles (Fig. 4A). In the tonsil, extensive areas of reticulate epithelium contained viral antigen. The viral distribution in the thymus was multifocal to diffuse and varied between lobules. Viral antigen was predominantly found in lymphocytes and in cells with dendritic morphology in the cortex. In the intestinal tract, viral antigen was most frequently seen in epithelium associated with lymphoid tissue. Besides epithelial cells on domes, small groups of epithelial cells
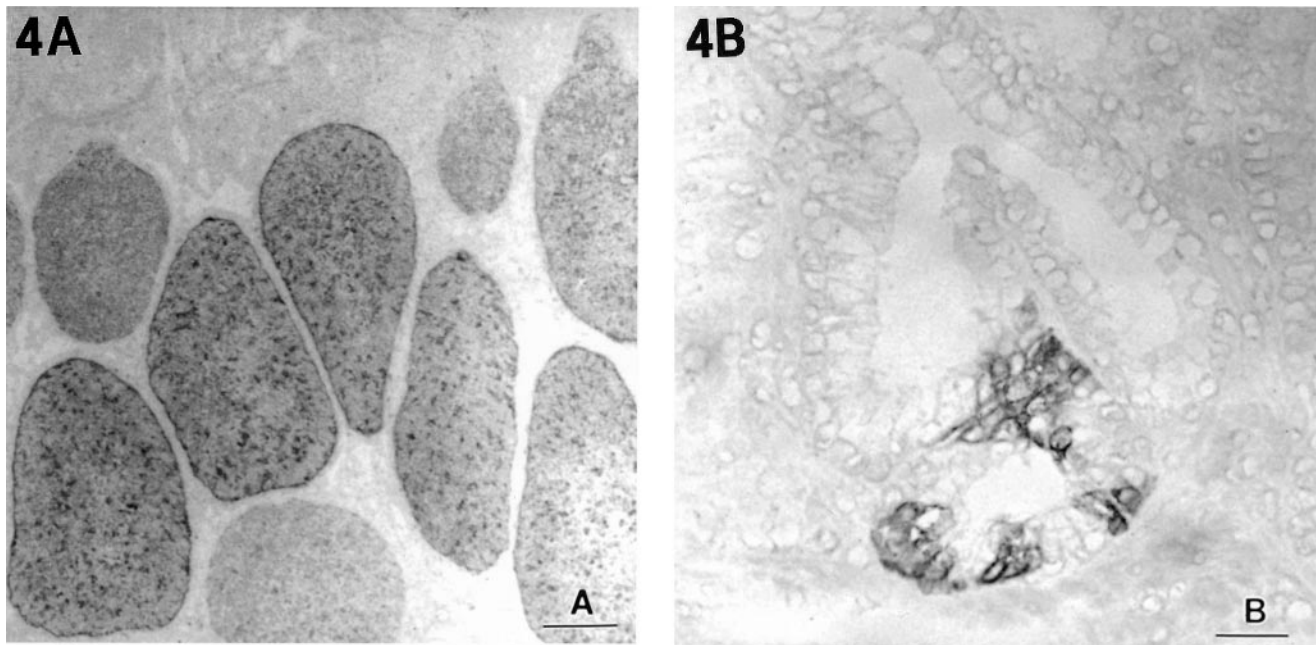
Figure 4. Distribution of viral antigen in acute infection at 6 dpi. **A,** presence of viral antigen is mostly restricted to lymphoid follicles. Ileal Peyer's patch. Calf 7, bar = 200 μm. **B,** groups of crypt epithelial cells contain viral antigen in the colon. Calf 9, bar = 25 μm.

were antigen positive in crypts in the small and large intestine (Fig. 4B). In individual calves, viral antigen was seen multifocally in the epithelium of the oral mucosa, the abomasum, the turbinates, and the trachea. Viral antigen was present in a bronchus-associated lymphoid follicle in the lung of calf 7.

At 9 and 13 dpi, viral antigen had been cleared from most tissues (Fig. 5A; Table 1). In a few lymphoid follicles in the ileal Peyer's patches, single, positive follicular dendritic cells and macrophages were present in 1 calf at 9 dpi. Cells with dendritic morphology in the thymic cortex were antigen positive in 1 calf at 9 dpi and in another at 13 dpi (Fig. 5B). Viral antigen was present in the wall of an arteriole in the spleen at 9 dpi and in the wall of medium-sized arterioles in the hilus of the retropharyngeal lymph node at 13 dpi (Fig. 5C). The cells in the periarteriolar infiltrate were negative for viral antigen.

Viral antigen was not detectable at any time after inoculation in tissues of the endocrine, urogenital, and central nervous system; liver, heart, and skin, or any other tissues of the 2 control calves necropsied.

Histological findings. In the PI calves, presence of viral antigen in tissues was not associated with tissue lesions.

In the acutely infected calves, mild depletion of a few lymphoid follicles in the tonsil and the retropharyngeal lymph node was seen in 1 of the calves at 3 dpi. At 6 dpi, increased cell death occurred in lymphoid follicles, but follicles were still large with the exception of calf 9 (Fig. 6). Lesions were more severe

in tonsils and retropharyngeal, mandibular, and mesenteric lymph nodes than in the jejunal and ileal Peyer's patches. Initially, single lymphoid follicles were affected; however, as time after infection progressed, an increasing number of follicles were involved.

At 9 dpi, there was marked depletion in all lymphoid tissues. Lobules of the tonsil were small (Fig. 7), the cortex in the lymph nodes was reduced in size, and few lymphoid follicles were present. The few lymphoid follicles present were large and active with numerous mitotic figures. Most lymphoid follicles were severely depleted in the Peyer's patches and lymphoid tissues of the large intestine. In the jejunal Peyer's patch, groups of lymphoblasts were present in a few lymphoid follicles. In 1 calf, the thymic cortex was multifocally depleted.

At 13 dpi, most lymphoid tissues had many large, active lymphoid follicles (Fig. 8A, 8B). In the jejunal Peyer's patch and in the gut-associated lymphoid tissue of the large intestine, the changes ranged from severely depleted to follicles that were variably repopulated with lymphocytes to normal lymphoid follicles. All lymphoid follicles in the ileal Peyer's patch were severely depleted, and the thymic cortex was moderately depleted in both calves (Fig. 8C, 8D). In 1 calf (calf 12), severe vasculitis was found in arterioles and small arteries at the hilus region of the mandibular, retropharyngeal, and parotid lymph nodes as well as in the myocardium (Fig. 8E). Lesions were characterized by
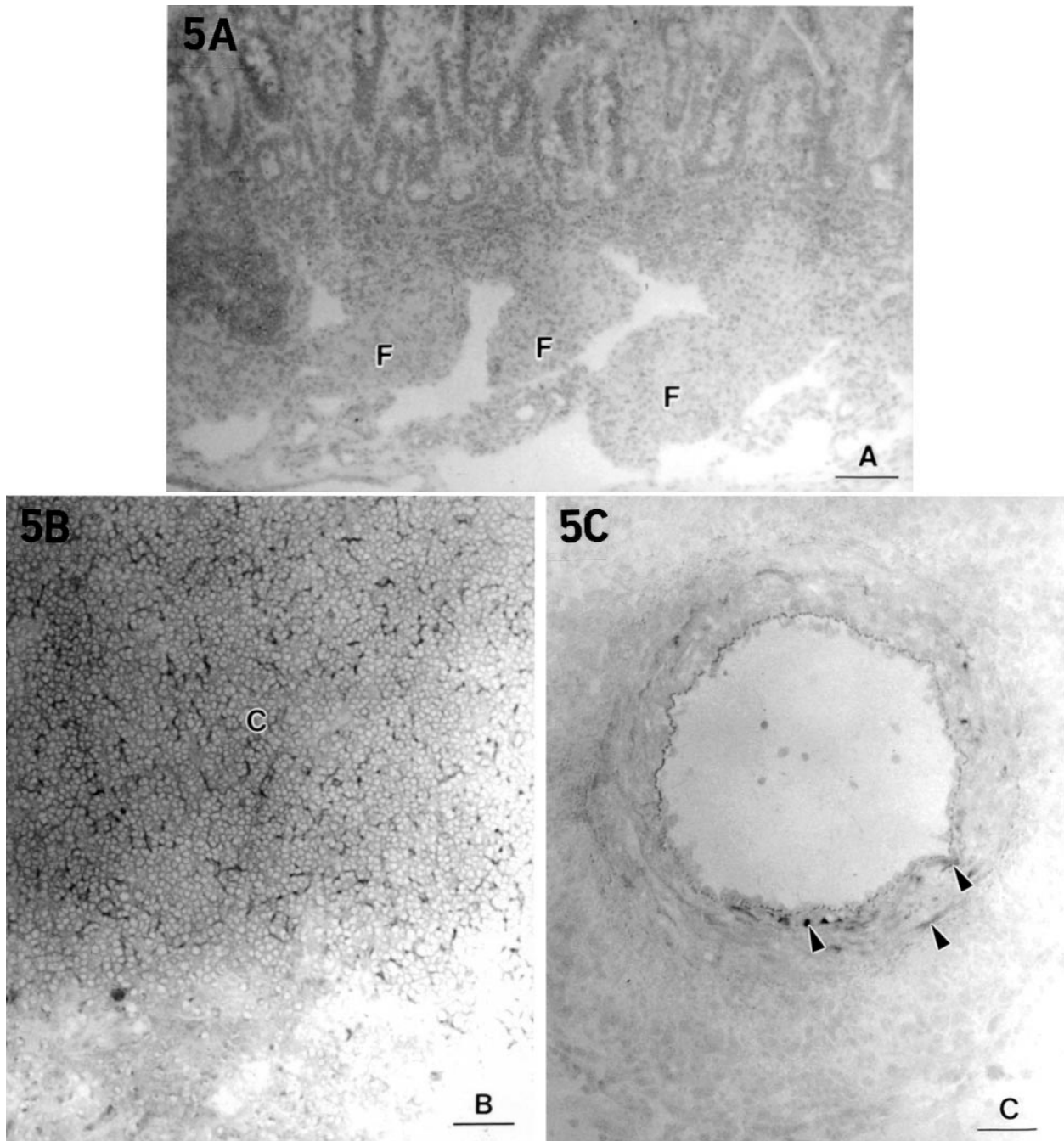
Figure 5. Distribution of viral antigen in acute infection at 9 and 13 dpi. **A,** there is no viral antigen in the severely depleted lymphoid follicles (F) of the ileal Peyer's patch of calf 11. Bar = 100 μm. **B,** many cells with dendritic morphology contain viral antigen in the thymic cortex (C) of calf 11. Bar = 50 μm. **C,** viral antigen (arrowheads) is present in the wall of a medium-sized arteriole in the hilus of the retropharyngeal lymph node of calf 12. Bar = 50 μm.

multifocal areas of fibrinoid necrosis and severe perivascular lymphohistiocytic infiltrates.

Discussion

Although antigen of the ncp BVDV2-RS886 was present in calves with both PI and acute infection, the distribution was different. In PI calves, viral antigen was widespread in all tissues and cell types as reported in the literature.[3] Glomerulonephritis and encephalitis, which have been described in some but not all cases of PI,[3,8] were not observed. In the calves examined in this study, there were no lesions associated with the presence of viral antigen.

In acute infection, the same virus (BVDV2-RS886) induced mild clinical signs, mild lymphopenia, and no thrombocytopenia and would thus be considered a
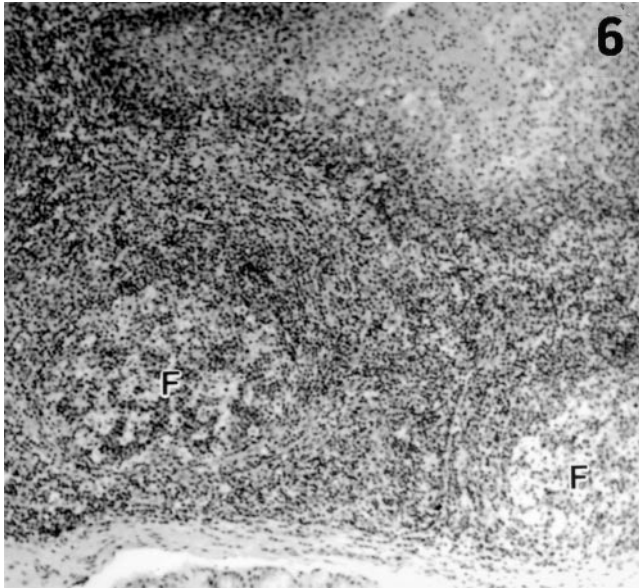
Figure 6. Lesions in the tonsil at 6 dpi in acute infection. There is markedly increased apoptosis in the large lymphoid follicles (F). Calf 7, paraplast section, HE, bar = 100 μm.
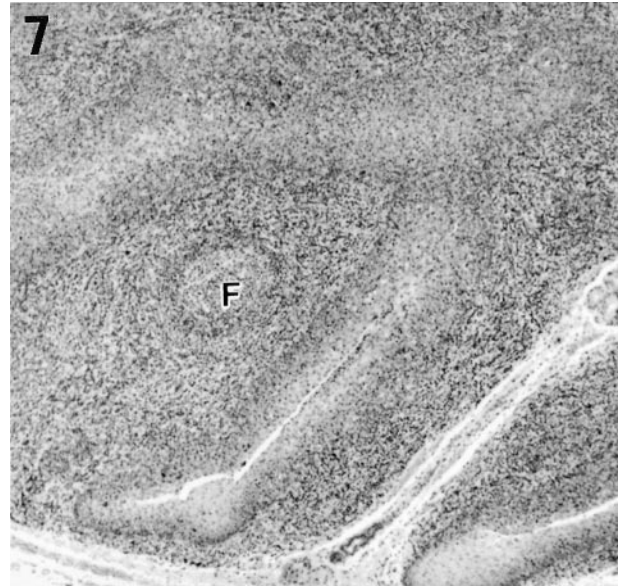

Figure 7. Lesions in the tonsil at 9 dpi in acute infection. The lobules of the tonsil are small. Few lymphoid follicles (F) are present. Calf 10, paraplast section, HE, bar = 200 μm.

low-virulence strain. The distribution and spread of virus was as reported for other low-virulence strains of BVDV2.[17] It was predominantly found in lymphoid tissues where it was present in large numbers of lymphocytes and cells with dendritic morphology in lymphoid follicles and the thymic cortex.[17,19] Compared with the distribution of viral antigen in the PI calves, BVDV2-RS886 in acutely infected calves was much more restricted to certain tissues, e.g., lymphoid tissues, and within the tissues to certain compartments, e.g., lymphoid follicles. Staining for viral antigen was much more intense in acute infection, indicating that larger amounts of antigen were present than in the PI calves.

The elimination of virus from most tissues, which was observed after 6 dpi in the acute infection, coincided with a severe loss of lymphocytes in lymphoid follicles and thymic cortex. The fact that lesions were only observed in acute infection, where the immune system recognizes the infecting BVDV, and not in PI, where the virus is not recognized as "foreign," suggests that the host's immune response contributes to the lesions in acute infection with ncp BVDV2 of low virulence.

T cell–mediated immune protection is mediated by cell destruction and thus lesions are an expected result. The balance between beneficial and harmful effects depends on the balance between spread of the virus infection and kinetics of the immune response.[28] Information regarding cell-mediated immunity in BVDV infections is fragmentary. Depletion of T lymphocyte subsets in acute BVDV infection revealed that deple-

tion of CD4+ T lymphocytes leads to prolonged viremia.[14] Depletion of CD8+ T lymphocytes had no effect on the duration of viremia, although antigen-specific cytotoxic lymphocytes can be demonstrated in the peripheral circulation.[2,14] These observations suggest that CD4+ T lymphocytes might be more important in BVDV infection. Although CD4+ T lymphocytes are commonly associated with helper functions, they can also be cytotoxic and induce apoptosis via the Fas/Fas-ligand system.[12] Apoptotic cell death is observed in lymphoid follicles in the early phase of acute BVDV infection.[16]

In acute BVDV infection, elimination of virus may benefit the host despite the marked depletion of lymphoid tissues because it prevents the widespread infection of tissues as seen with more virulent BVDV2 strains.[11,16,23] If virus elimination is not complete, the depletion of lymphoid tissues and immunosuppression might explain the detection of BVDV over extended time periods after infection, as is observed in some acute infections.[7]

The comparison between the effects of an ncp BVDV strain in PI versus acute infection indicates that ncp BVDV strains from PI cattle cannot in general be considered as avirulent, although they may not cause lesions in the PI animals. Thus, shedding of these viruses poses not only a problem for pregnant cows by causing intrauterine infection of the fetus but may also lead to acute infection and immune suppression of other herd members.
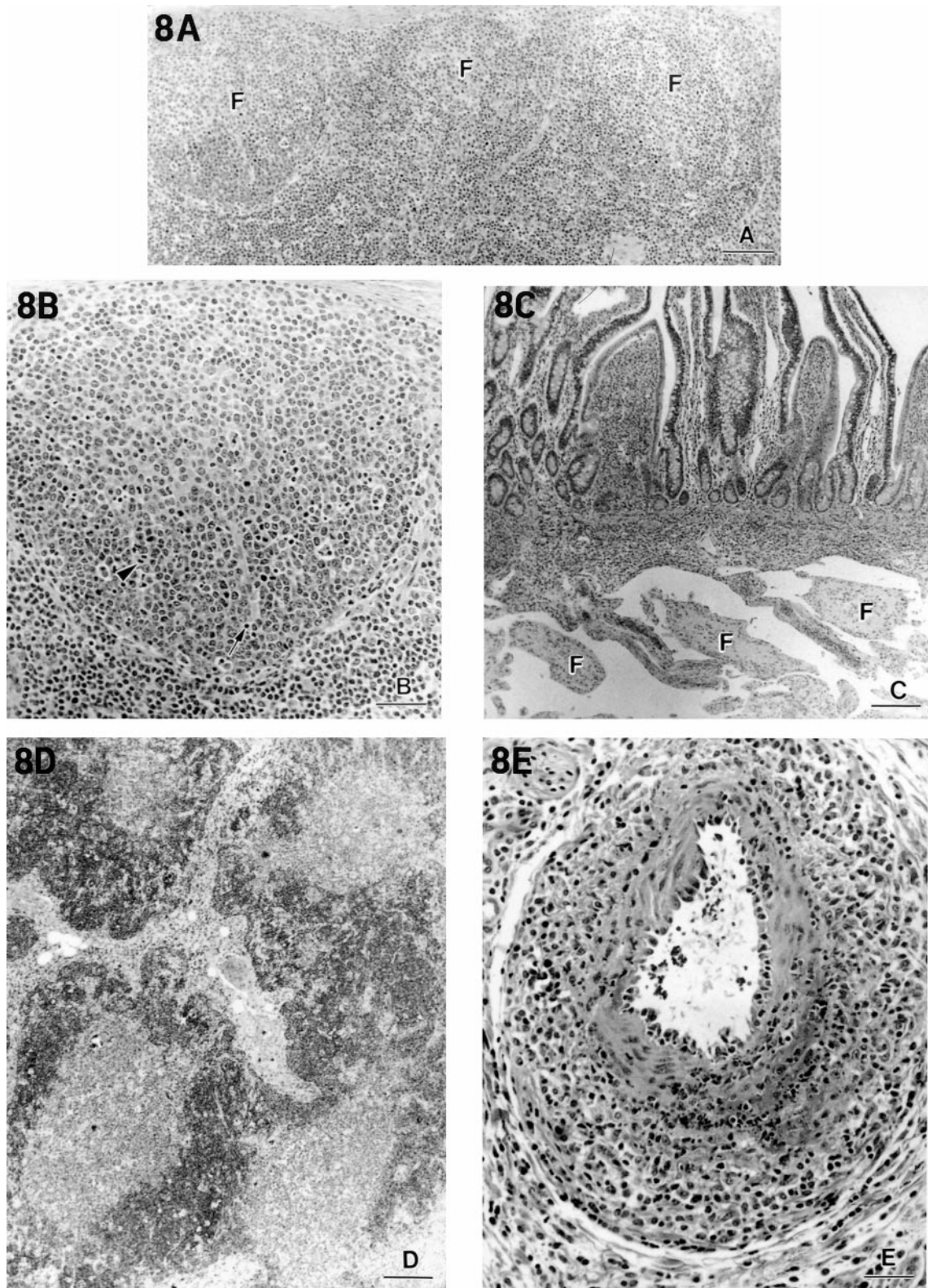
Figure 8. Morphological findings at 13 dpi in acute infection (calf 12, paraplast section, HE). **A,** numerous large lymphoid follicles (F) are present in the mesenteric lymph node. Bar = 100 μm. **B,** the higher magnification of a lymphoid follicle reveals high numbers of lymphoblasts, numerous mitotic figures (arrowhead, example), and many apoptotic bodies (arrow, example). Bar = 50 μm. **C,** lymphoid follicles (F) in the ileal Peyer's patch are severely depleted. Bar = 200 μm. **D,** there is a multifocal, moderate depletion of the thymic cortex. Bar = 200 μm. **E,** fibrinoid necrosis and perivascular lymphohistiocytic infiltrates in an arteriole in the myocardium. Bar = 50 μm.

Sources and manufacturers

a. Monoclonal antibody (mab) BZ81, obtained from Julia F. Ridpath, National Animal Disease Center, USDA-ARS, Ames, IA.
b. Tween-20, Bio-Rad Laboratories, Richmond, CA.

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